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14. ABSTRACT The goal of this project is to determine the role of ERK/MAP kinase phosphorylation of the RUNX2 transcription factor in the metastasis of prostate cancer cells. In the third budget year, we achieved the following: a. Generation of retrovirus and lentivirus vectors expressing WT RUNX2 and S301A, S319A phosphorylation-deficient RUNX2 and S301E, S319E phosphomimetic Runx2 mutants. Isolation of stable PC3 and LnCaP cell lines expressing WT and mutant RUNX2. b. Demonstration that phosphorylation-deficient RUNX2 has reduced ability to stimulate in vivo tumor formation when PCa cells are implanted into immunodeficient mice. These results continue to support our overall hypothesis that RUNX2 phosphorylation is a critical determinant of tumorigenicity and metastasis of prostate tumor cells.					
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Epigenetic Control of Prostate Cancer Metastasis: Role of Runx2 Phosphorylation

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INTRODUCTION

Bone metastasis leading to the formation of mixed osteoblastic and osteolytic lesions is seen in ~80% of men with metastatic prostate cancer (PCa)¹. Little is known concerning the cellular signals controlling the metastatic behavior of the primary tumor. The presence of the RUNX2 transcription factor in prostate cancer cells has been related to invasive/metastatic behavior²⁻⁵. This project is based on preliminary studies suggesting that metastatic behavior of prostate tumors requires activation of the ERK/MAP kinase pathway, which phosphorylates the RUNX2 transcription factor. Previous work from our laboratory had shown that S301, S319 phosphorylation of RUNX2 is critical for its transcriptional activity in bone⁶, and we hypothesized that these sites are also important for RUNX2-dependent metastatic activity. During the first year of this grant, efforts were focused on determining if RUNX2 phosphorylation is related to metastasis-associated cell properties. In addition, preliminary studies were conducted with prostate cancer tissue microarrays to determine if there is a relationship between RUNX phosphorylation levels and tumor outcome. In year 2, we initiated studies related to RUNX2 phosphorylation and epigenic changes in metastasis-associated genes, expanded studies on the role of RUNX2 phosphorylation in stimulating in vitro cell migration and invasion using non-tumorigenic cells, began in vivo metastasis experiments and completed a large tumor tissue microarray study to confirm the association of P-RUNX2 with prostate cancer in human samples. In year 3, we completed a study showing that adenovirus transduction of PC3 cells with wild type RUNX2 stimulated in vivo tumor formation and angiogenesis in immunodeficient mice while phosphorylation-deficient RUNX2 was inactive. Combined results of our studies are being incorporated into a manuscript that will be submitted within the next month.

BODY

Project Tasks: Accomplishments over the past year are listed according to original tasks described in the Statement of Work.

Task 1: Establish the relationship between MAP kinase signaling, Runx2 phosphorylation and transcriptional activity in normal prostate epithelial and PCa cells and determine how Runx2 phosphorylation controls VEGF gene expression.

a. Levels of total and P-ERK, total and P-Runx2 and Runx2-dependent activation of metastasis-associated genes will be compared in normal human prostate epithelial cells, K-ras-transformed prostate epithelial cells, non-metastatic LNCaP cells and three metastatic cell lines (PC-3, C4-2B, VPCa).

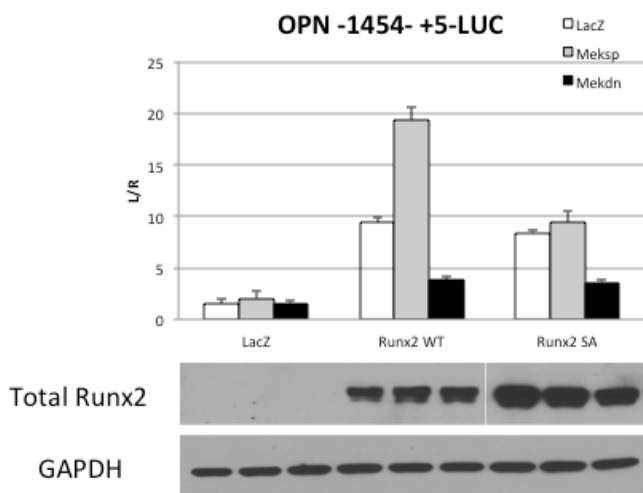
Comparison of cell lines showed that RUNX2 mRNA and protein levels were greatly elevated in the highly metastatic cell line, PC3, with progressively lower levels in C4-2B and LNCaP cells. These studies were completed in year 2 and were reported last year.

b. Specific inhibitors of ERK/MAPK, Src and PI3K/AKT pathways as well as siRNA inhibition and overexpression of key pathway intermediates will be used to assess the relative importance of each signal transduction pathway in expression of metastatic genes.

These studies are still in progress.

c. Runx2-responsive regions of the proximal Vegf promoter will be identified using deletion/mutation analysis, functional assays and chromatin immunoprecipitation (ChIP).

Fig 1. Induction of Spp1 promoter activity by WT and S301A/S319A mutant Runx2. PC-3 cells were transfected with a 1.45 kb Spp1 promoter-luciferase reporter gene, renilla luciferase (for normalization of transfection) wild type or mutant Runx2 expression vectors, with or without a constitutively active (Meksp) or dominant-negative MAP kinase expression vector (Mekdn). Luciferase activity was measured after 48h. Runx2 protein levels are also shown.



For these experiments, we decided to study the matrix metalloproteinase 13 (Mmp13) and the osteopontin (Spp1) promoters because their regulation by RUNX2 has been more extensively studied relative to VEGF and is more directly related to the invasive behavior of cells^{4,7}. Studies with MMP9 were shown last year. We now show that wild type Runx2, but not a phosphorylation-deficient S301A/S319A mutant (Runx2-SA) can stimulate Spp1 gene expression and promoter activity (Fig. 1).

d. ChIP assays will be used to resolve whether binding of P-ERK to Vegf chromatin requires bound Runx2.

These studies are being conducted with the MMP13 and Spp1 genes and will be completed in the coming year.

e. Use wild type Runx2, S301A/S319A (non-phosphorylated) or S301E/S310E (constitutively active) Runx2 mutants to determine whether Runx2 phosphorylation is necessary for histone phosphorylation, acetylation and activation of Vegf transcription. These studies are also in progress using Mmp13 and Spp1 promoter regions.

Task 2: Determine if Runx2 phosphorylation is necessary for *in vitro* and *in vivo* proliferative, invasive and metastatic behavior of PCa cells stably transfected with wildtype Runx2 or Runx2 phosphorylation site mutants.

a. MLV-based retrovirus vectors will be developed that express β -galactosidase (negative control), wild type Runx2, S301A/S319A (non-phosphorylated) or S301E/S310E (constitutively active) Runx2 mutants. As described last year, retrovirus and lentivirus vectors were constructed to stably introduce wild type and mutant RUNX2 into cells. Both vectors are being used to develop stable cell lines using LNCaP and PC3 cells that are in the process of being characterized. Once this step is completed, these cells will be used to examine the effect of Runx2 phosphorylation on long-term tumor cell survival and metastasis to bone and other tissues *in vivo*. These studies will be completed in the final year of this project.

We also developed adenovirus vectors to express wild type and S301A/S319A mutant RUNX2. These vectors were used for analysis of metastasis-associated cellular activities in tissue culture and tumor growth after *in vivo* cell implantation (see below).

b. LNCaP cells with low intrinsic invasive/metastatic activity and Runx2 levels will be transfected with retrovirus vectors and stable lines will be isolated expressing β -Gal, wild type Runx2, S301A/S319A or S301E/S310E Runx2 mutants.

Initial attempts to develop stable lines of PC3 and LNCaP cells expressing β -galactosidase (negative control), wild type Runx2, S301A/S319A (non-phosphorylated) or S301E/S310E (constitutively active) Runx2 were not

successful. As described above, we are now pursuing the approach of using lentivirus vectors, which can be grown to higher titers and have higher transduction efficiencies. These vector properties will allow us to make stable luciferase-expressing RUNX2 lines of PCa cells having low (LNCaP) and high (PC3) basal metastatic activity as well as non-tumorigenic prostate cells (RWPE1 cells). These cells will then be used to measure the role of RUNX2 phosphorylation on in vivo metastasis studies which require long-term stable expression of the RUNX2 protein.

As an alternative approach to determine if RUNX2 phosphorylation is important for in vitro cell activity, adenovirus vectors encoding wild type and S310A/S319A RUNX2 were used to show that wild type, but not phosphorylation-deficient RUNX2 could stimulate metastasis-associated gene expression (MMP9, VEGF and osteopontin-OPN), angiogenesis, cell migration and invasion in vitro. These studies, which were included in last year's report, used both PC3 PCa cells and RWPE1 cells, an immortalized human prostate epithelial cell line that is not tumorigenic and has low in vitro migration and invasive activity. In both prostate-derived cell lines, wild type RUNX2 was shown to stimulate metastasis-related gene expression (induction of osteopontin, Spp1; vascular endothelial growth factor, Vegf; and matrix metalloprotease 9, MMP9), while a phosphorylation resistant RUNX2 mutant had greatly reduced activity.

c. In vitro migratory activity of stable LNCaP lines will be compared. As reported last year, PC3 and RWPE1 cells were transduced with AdLacZ, AdRUNX2wt or AdRUNX2 S301A/S319A mutant and plated onto glass slides. A central line of cells was removed using a Pasteur pipette and we measured the ability of cells to migrate into the cell-free area. WT RUNX2 clearly stimulated migration of both cell types, while migration of cells transduced with the phosphorylation-deficient mutant RUNX2 was similar to that of control cultures.

d. The in vitro invasive activity of LNCaP lines will be compared using a matrigel invasion assay. Also reported last year, wild type RUNX2 stimulated migration of PC3 and RWPE1 cells across a Matrigel_{TM} membrane, a cell culture model of tumor invasion. In contrast, a phosphorylation-deficient RUNX2 mutant had no activity in this assay.

f. In vivo proliferative activity of LNCaP cell lines will be measured after subcutaneous and intrafemoral implantation into immunodeficient mice. We recently examined the acute effects of RUNX2 on *in vivo* growth of tumors from implanted PC3 cells. PC3 cells stably expressing a firefly luciferase reporter (PC-luc cells) were transduced with LacZ, Runx2-WT or Runx2-SA adenovirus vectors to give equivalent amounts of Runx2 protein (Fig. 2B), suspended in Matrigel_{TM} and subcutaneously implanted into immunodeficient mice. Tumor development was monitored over a 16-day period by measuring whole-body luciferase activity. At day 17, mice were sacrificed and tumors were weighed and used for RNA analysis and immunohistochemistry. Wild type Runx2 significantly increased tumor development as measured by *in vivo* luminescence (Fig 2A,C) and tumor size/weight at sacrifice (Fig. 2D,E). In contrast, tumors formed by cells transduced with the Runx2-SA virus were not significantly different from LacZ controls. Similar to cell culture results, tumors from Runx2-WT-treated cells expressed higher levels of VEGF, MMP9 and SPP1 mRNAs relative to LacZ controls while levels were generally lower with Runx2-SA transduction (not shown). Visual inspection of wild type Runx2 tumors suggested possible increases in vascularization relative to controls or Runx2-SA samples. To verify this, histological sections from cells transduced with wild type RUNX2 stained positively with antibody to the vascular markers, von Willebrand factor and CD31 (not shown).

These studies support our hypothesis that Runx2 phosphorylation is necessary for in vivo tumor formation.

g. In vivo metastatic activity of LNCaP cell lines will be measured using an orthotopic (intraprostate implantation) model.

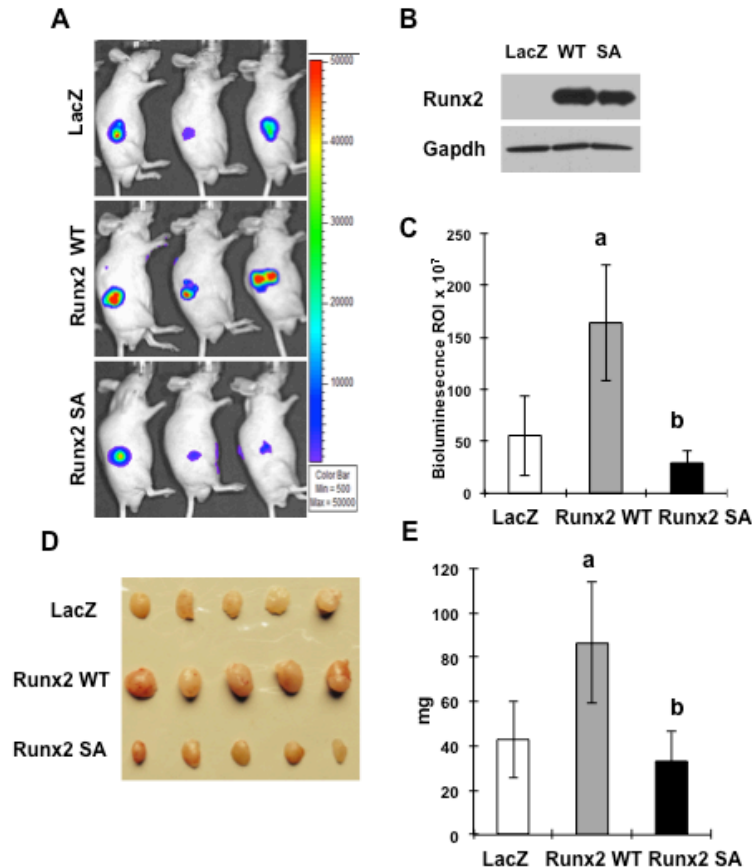
To be completed in the final no-cost extension year of this project.

h. In vivo metastatic activity of LNCaP cell lines will be measured using an intracardiac injection model.

To be completed in the final no-cost extension year of this project.

Task 3: Correlate phosphorylated (S319-P) Runx2, total Runx2 and P-ERK immunoreactivity with tumor outcome using a panel of human PCa tissue microarrays (TMAs) composed of normal prostate tissue, benign prostatic hyperplasia, prostatic intraepithelial neoplasia and prostate cancer.

Fig.2. Role of RUNX2 phosphorylation in tumor growth in vivo. PC3-luc cells were transduced with LacZ, RUNX2-WT or RUNX2-SA adenovirus vectors to give equal protein levels as measured by Western blot (B) and subcutaneously implanted in immunodeficient mice. In vivo tumor growth was monitored by bioluminescence imaging shown at d16 (A,C). At d17, tumors were harvested, photographed/weighed (D,E). von Willebrand factor and CD31 were measured by immunohistochemistry and mRNA levels were measured by RT-PCR (not shown). N = 9/group



a. TMAs provided by the Michigan Prostate Center SPORE will be immunostained using total and P-Runx2-specific antibodies as well as total and P-ERK antibodies.

b. Patterns of immunoreactivity will be correlated with tumor history; statistical models will be used to evaluate the predictive values and relationship to clinical parameters for each marker.

During year 2, we completed a comprehensive TMA analysis in collaboration with Dr. Guiseppe Panone (University of Foggia, Italy) who developed TMAs of prostate tissue that were stained with a P-RUNX2-specific antibody developed in the the project laboratory (antibody specifically recognizes RUNX2-S319-phosphate). TMAs included prostate diseases from 129 caucasian patients. Details of this study were reported last year.

These studies indicated that P-RUNX2 is an excellent biomarker for early and late stages of prostate cancer.

KEY RESEARCH ACCOMPLISHMENTS- YEAR 3

a. Generation of PC3-luciferase cell lines stably transduced with lentivirus and retrovirus vectors expressing WT RUNX2 and S301A, S319A phosphorylation-deficient RUNX2. These cell lines will be used for in vivo metastasis and bone transplantation

experiments in the coming year.

b. Demonstration that phosphorylation-deficient RUNX2 has reduced ability to stimulate in vivo tumor formation and tumor angiogenesis when compared with wild type RUNX2.

REPORTABLE OUTCOMES

Previously reported outcomes:

The following RUNX2 expression vectors were generated :

AdRUNX2 WT- expresses wildtype murine RUNX2 at high levels in prostate cancer cells.

AdRUNX2 Mut- expresses S301A, S319A phosphorylation-deficient RUNX2 at high levels in PCa cells.

Lenti-RUNX2 WT- expresses wildtype murine RUNX2 at high levels in prostate cancer cells.

Lenti-RUNX2 Mut- expresses S301A, S319A phosphorylation-deficient RUNX2 at high levels in PCa cells.

Results from this project have been reported in abstract form and presented at the 11th International Conference of Cancer-Induced Bone Disease, Chicago, IL, November 30-December 3, 2011.

Zhao Z, Ge C, Pannone G, Bufo P, Santoro A, Sanguedolce F, Tororella S, Mattoni M, Papagerakis P,

Papagerakis S and **Franceschi RT**. RUNX2 phosphorylation as a prognostic marker of metastatic disease in prostate cancer. 11th International Conference of Cancer-Induced Bone Disease Proceedings (2011) Abst P145.

We are in the final stages of preparing a full manuscript describing this work that will be submitted within the next month.

CONCLUSIONS

Thus far in this project, we made good progress for all 3 tasks. Specifically, we clearly showed that the RUNX2 transcription factor is able to induce the expression of metastasis-associated genes and increase in vitro migratory, angiogenic and invasive properties of prostate cancer cell lines. Furthermore, RUNX2 must be phosphorylated to stimulate these activities. Significantly, in the past year we also found that wild type RUNX2 can stimulate in vivo tumor formation by implanted PC3 cells, but that this activity is also requires intact phosphorylation sites in the RUNX2 protein. A tissue microarray study with a total of 129 patient samples showed a good correlation between the presence of RUNX2-S319-P in prostate tissue and neoplastic activity with minimal staining observed in benign prostate hyperplasia or prostatitis. Efforts in the coming year will be focused on examining the importance of RUNX2 phosphorylation in tumor metastasis in vivo using orthotopic as well as intracardiac administration of PCa cells. Lastly, we will examine the mechanism through which RUNX2 phosphorylation controls the activity of metastasis-related genes. A no cost extension for this project was approved to complete this work (Dates 04/01/14 - 03/31/2015).

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